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MECHANISM OF ACTION OF TETANUS TOXIN

Annual Report

Mark S. Klempner, M.D.

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The mechanism by which tetanus toxin (TT) inhibits the release of neurotransmitters from neurons is unknown. Since secretion of lysosomal contents from monocytes/macrophages (MOs) appears to be similar to neurosecretion, we examined the effects of TT on MO secretion. In this system, TT-treated MOs caused a dose-dependent inhibition of lysozyme secretion in response to a calcium ionophore - A23187. Inhibited secretion was additionally demonstrated in TT-treated MOs when challenged with other soluble stimuli, phorbol myristate acetate (PMA) and formyl-leucyl-methionyl-phenylalanine (f-MetLeuPhe). Treatment with TT also caused inhibition of superoxide generation (O_2-) in response to A23187, f-MetLeuPhe, PMA and opsonized zymosan.

To explore the intracellular mechanism of inhibition of secretion by TT, cellular

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19. Abstract (Cont.)

calcium homeostasis was examined. Using Quin-2 as a calcium probe for cytosolic calcium, we found that the inhibition of secretion by MO was not directly linked to calcium homeostasis, even though TT inhibits the rise of cytosolic calcium in response to low concentrations of ionomycin. Since TT inhibited secretion in response to PMA without a detectable increase of cytosolic calcium and TT did not alter cytosolic calcium in response to PMA, we explored whether TT had an effect on cellular protein kinases.

In unstimulated MO, cytosolic protein kinase C (PKC) was inhibited in a dose-dependent manner. The inhibition was not on the basis of redistribution of PKC into membrane associated fraction. The inhibition of PKC by TT is specific since PKA, another kinase important in stimulus-response coupling, was similar in control (C-) and TT-treated MOs. Moreover, heat-inactivation of TT and removal of TT by immunoabsorption resulted in similar PKC activity in C- and TT-treated MOs.

We have developed an in vivo mouse model of lethal generalized tetanus to examine the effect of TT on cellular kinases. TT-intoxication resulted in diminished cytosolic PKC activity in spinal cord of mice given 50x minimal lethal dose of TT. Brain PKC activity from the same animals was similar between C- and TT-intoxicated mice. The difference in PKC activity was specific since PKA activity was similar in C- and TT-treated animals.

We have demonstrated that incubation of PMN lysosomes with TT caused an augmented uptake of Ca^{2+}. To explore the interaction of TT on PMN lysosomes, we have embarked on the purification of the PMN lysosomal Ca^{2+}-ATPase. Major progress in the purification of the lysosomal Ca^{2+} uptake pump has been achieved.

SUMMARY PAGE

The mechanism by which tetanus toxin (TT) inhibits the release of neurotransmitters from neurons is unknown. Since secretion of lysosomal contents from monocytes/macrophages (MOs) appears to be similar to neurosecretion, we examined the effects of TT on MO secretion. In this system, TT-treated MOs caused a dose-dependent inhibition of lysozyme secretion in response to a calcium ionophore - A23187. Inhibited secretion was additionally demonstrated in TT-treated MOs when challenged with other soluble stimuli, phorbol myristate acetate (PMA) and formyl-leucyl-methionyl-phenylalanine (f-MetLeuPhe). Treatment with TT also caused inhibition of superoxide generation (O_2-) in response to A23187, f-MetLeuPhe, PMA and opsonized zymosan.

To explore the intracellular mechanism of inhibition of secretion by TT, cellular calcium homeostasis was examined. Using Quin-2 as a calcium probe for cytosolic calcium, we found that the inhibition of secretion by MO was not directly linked to calcium homeostasis, even though TT inhibits the rise of cytosolic calcium in response to low concentrations of ionomycin. Since TT inhibited secretion in response to PMA without a detectable increase of cytosolic calcium and TT did not alter cytosolic calcium in response to PMA, we explored whether TT had an effect on cellular protein kinases.

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The mechanism by which tetanus toxin (TT) inhibits the release of neurotransmitters from neurons in unknown. Since secretion of lysosomal contents from monocytes/macrophages (MOs) appears to be similar to the release of synaptosomal contents from neural cells, we examined the effects of TT on MO secretion. We have described that human MOs may be a readily accessible, model system for study of the biochemical basis of TT intoxication. In this system, TT-treated MOs (1.25 ug/ml) stimulated by A23187 secreted significantly less lysozyme (27.2% $\pm$ 3.2%) than did control cells (48.6% $\pm$ 6.8%; 44.1% inhibition, $p < 0.001$). TT inhibition of A23187-stimulated release of lysozyme from MOs was dose-dependent. MO secretion in response to phorbol myristate acetate (PMA) and f-met-leu-phe (fMLP) was also inhibited by TT. Heated TT, which has no in vivo toxicity, also failed to inhibit MO section. The inhibition by TT required holotoxin. C- and B-fragments produced no appreciable effect on secretion (1). We examined the effect of TT on another MO cell function and found that TT inhibits production of superoxide in response to A23187, PMA, fMLP and serum-opsonized zymosan (figures 1,2). Since fMLP receptors and components of the NADPH oxidase system are associated with granules in human phagocytic cells, and when stimulated both of these increase in number on the plasma membrane, we evaluated the effect of TT on fMLP receptor expression and recruitment. In the basal state, fMLP receptors were similar in C-MO (116,867 $\pm$ 30,391) and the TT-treated MO (98,054 $\pm$ 25,277), N=6. Preliminary data indicates that TT-treated MO recruit significantly fewer fMLP receptors than C-MOs (79.9% inhibition). These data suggest that TT inhibits recruitment of NADPH oxidase components and, thus, have inhibited superoxide generation when stimulated by PMA. Because secretory events are associated with changes in cytosol free calcium, we examined the effect of TT on MO calcium homeostasis. Using Quin-2 as a probe to measure the cytosolic ionized calcium concentrations, we found that the inhibition of secretion by MO was not directly linked to Ca^{2+} homeostasis, even though TT inhibits the rise of cytosolic calcium in response to low concentrations of ionomycin. This conclusion is based on the following: 1. In response to ionomycin concentrations that induce secretion, TT-treated MO and control MO both attain similar cytosolic calcium concentrations; 2. In response to ionomycin 0.1 uM, there was no secretion with a similar increment in cytosolic free [Ca^{2+}] from TT-treated and control MO; 3. TT inhibition of MO secretion in response to PMA was independent of extracellular calcium, and; 4. TT inhibited secretion in response to PMA without a detectable increase of cytosolic calcium and TT did not alter cytosolic calcium in response to PMA (2).

We next wondered whether TT might interfere with protein kinase C (PKC), an enzyme important in stimulus-secretion coupling. PKC is activated by a rise of cytosolic calcium and diacylglyceral, both of which are generated during receptor-ligand activation of phospholipase C (3,4). The hypothesis that TT may interfere with PKC activity was based on the observations that MO secretion in response to PMA, a stimulus which activates PKC (1,2), was inhibited by TT and the inhibition of secretion by TT was not directly linked to cytosolic calcium homeostasis (2). We measured PKC activity in non-activated MO treated with or without TT.

PKC activity, as defined by transfer of P^{32} from γ-P^{32} -ATP to histone in the presence of PMA, added phosphatidylserine (PtS) and Ca^{2+} minus phosphorylation without these cofactors, was diminished in TT-treated MO (Figure 3). The diminished activity could not be explained by redistribution of PKC to a membrane associated compartment since PKC activity in the triton X-100 solubilized particulate fraction was also diminished in TT-treated MO (Figure 3A). Inhibition of PKC by TT is dose-dependent (Figure 3B). For the highest tested dose of TT (17.0 ug/ml), a 70% inhibition of PKC was observed. The degree of inhibition of PKC by TT is comparable to the ~70% inhibition of PKC observed by Gerard & McPhail et al (8) in neutrophils treated with high concentrations of a kinase inhibitor, C-I.

Further evidence that we are measuring PKC activity is provided by the activation of PKC by different phorbols. PKC activity in the absence of any phorbol was 6.5 p-mole per 10 min for C-MO and 3.6 p-mole per 10 min for TT-treated MO. α-Phorbol didecanoate (PDDC), which does not stimulate PKC in other systems (9,10), does not stimulate macrophage PKC and does not stimulate lysozyme secretion (Table 1). β-PDDC, which has an intermediate potency to activate PKC in other systems (9), resulted in an intermediate activation of PKC and lysozyme secretion when compared to PMA at 6 uM (10-fold less). TT-treatment (1.7 ug/ml) inhibited both secretion and PKC in MO stimulated by two active phorbols (B-PDDC and PMA, Table 1). PKC utilizes the gamma phosphate of ATP to phosphorylate cellular proteins. The TT inhibitable protein kinase activity also demonstrated the same nucleotide specificity. Compared to γ-P^{32} -ATP, phosphorylation utilizing GTP/γ-p^{32} - GTP was 11.1% $\pm$ 3.5% and phosphorylation utilization ATP/α-p^{32} -ATP was 1.2% $\pm$ 4.9%, N=4. Similar results were observed for TT-treated kinase activity.

Protein kinase A (c-AMP dependent) activity from cytosolic preparations was similar in C-MO and TT-treated MO (Figure 4). In contrast, cytosolic preparations from the same cells demonstrated diminished PKC activity after TT-treatment (47.8% $\pm$ 10.5% of PKC activity in C-MO).

Additional experiments intoxicating MO with highly purified preparation of TT also caused diminished PKC activity (Figure 5A). Heat-inactivation of TT resulted in loss of biological activity, reversal of its ability to inhibit MO secretion (1) and in similar PKC activity in C-MO and MO treated with heat inactivated TT (Figure 5B). The ability of an active preparation of TT to inhibit MO PKC activity was removed by specific immunoabsorption (Figure 5B).

The data presented demonstrate that TT-treated MO have diminished PKC activity. This decreased phosphorylating enzyme activity was specific for PKC since PKA activity was similar in C-MO and TT-treated MO. We do not know the mechanism by which TT modifies PKC activity. However, it is not caused by a generalized reduction of protein synthesis since cytosolic protein concentration from C-MO and TT-treated MO did not differ significantly, 273.0 $\pm$ 22.9 ug per 2 x 10^7

cells versus 254.1 ± 21.1 ug per 2 x 10^7 cells respectively, N=30 (Figure 3A). When adjusted for protein concentration, PKC (expressed as p-mole/mg-protein/10 min) was 3716.0 for C-MO and 2296.5 for TT-treated (1.7 ug/ml) MO. In addition, increasing the substrate concentration (ATP, histone) had no effect on the diminished PKC activity in TT-treated MO. At low substrate concentration (5 uM ATP, 0.15 mg histone per ml) PKC activity from TT-treated MO was 61.5% ± 5.3% of C-MO and with higher substrate concentration (10 uM ATP, 1.5 mg histone per ml) PKC activity from TT-treated MO was 53.1% ± 8.9% of C-MO, N=3.

Protease-activated kinase II (PAK II) is a kinase that shares with PK-C similar molecular weight and activation by diolein and PtS (11). Although we have not excluded the possibility that our observations are due to inhibition of PAK II, several features make this less likely. Almost all of the reported experiments were performed with 8.5 uM of ATP, an amount which is below the Km of PAK II for ATP (40 uM) (11). In addition, compared to no added Ca^{2+}, 1 mM Ca^{2} increased kinase activity by 1.3-fold (N=14), a finding which is similar to that reported for PK-C (1.47-fold) but not PAK II (11).

Preliminary experiments suggest that intracellular processing of holotoxin may be required for its ability to inhibit MO PKC activity. Cytosolic preparations from C-MO preincubated with TT holotoxin (0.3 ug per ml) for 5 min prior to initiation of phosphorylation by the addition of ATP/ γ-P^{32} -ATP had similar PKC activity compared to untreated cytosolic preparations. In contrast the cytosolic preparations from TT-treated MO had diminished PKC activity (data not shown). Since PKC appears to be important in stimulus-secretion coupling (13,14) and has been shown to localize around granules in phagocytic cells from bone marrow (15) and in both neural cell bodies and axon terminals (16), its diminished activity in TT-treated MO may be causally related to the inhibition of secretion by TT.

To explore whether PKC activity is diminished in vivo, we examined the PKC activity from neural tissue of mice intoxicated with TT. Mice manifesting generalized tetanus after receiving a 50x lethal dose of TT were sacrificed and PKC was assayed from homogenized brain and spinal cord by measuring the transfer of γ-^{32}P-ATP to histone. PKC activity (pmole/mg-protein/min) was similar in brain cytosol of control (C) and TT-intoxicated mice, (378.2 ± 107 and 354.4 ± 119 respectively) (Figure 6). Brain membrane associated PKC activity was also similar for C- and TT-intoxicated mice. Cytosolic PKC activity in the spinal cord was depressed in TT-intoxicated mice, 87.3 ± 25 compared to control animals, 182.6 ± 47, $p<.02$, N=7 (Figure 6). Membrane associated PKA activity in the spinal cord was similar in C- and TT-intoxicated mice, (21.4 ± 17.5 vs 20.4 ± 15.2 pmole/ml protein/min). Cytosolic PKC activity from brain and spinal cord was similar for C- and TT-intoxicated mice. Thus, the ability of TT to interfere with cytosolic PKC activity in the spinal cord but not the brain may be related to the clinical manifestations of tetanus intoxication.

One of the other major goals of the contract is to examine the interaction of tetanus toxin with a novel Ca^{2+}-ATPase localized in the lysosomes of phagocytic cells. We have previously shown that tetanus holotoxin and tetanus B-fragment augment PMN-lysosomal calcium uptake (16). Despite the finding that the inhibition of secretion by TT was not directly linked to cytosolic calcium homeostasis,the rise of cytosolic [Ca^{2+}] was inhibited in TT-treated MO when substimulatory concentrations of ionomycin were used. In an attempt to define whether the augmentation of Ca^{2+} uptake by PMN lysosomes is specific, two different types of experiments have been performed. Since the TT preparation obtained from Cal-Biochem has several protein bands (MW 150 Kdal to 80 Kdal), visible in coomassie blue-stained polyacrylamind gels, TT was obtained from the Massachusetts State Laboratory. This preparation has a single band on PAGE under non-reducing conditions. When lysosomes were treated with this TT-(3ug/ml), these lysosomes had significantly greater ATP-dependent Ca^{2+} uptake compared to untreated lysosomes (Control:C, Table 2).

We also examined whether the interaction of TT with PMN lysosome caused an irreversible augmentation of Ca^{2+} uptake. Ca^{2+} uptake experiments were performed on lysosomes in which TT was removed after pre-incubation. C-lysosomes and TT-lysosomes preincubated at 37°C or 4°C were subsequently pelleted by centrifugation (epperdorf, 12,000 g x 2 min). The supernatants were removed, the pellets were washed twice and finally resuspended in buffer for calcium uptake. We found that washing of the TT-lysosomal pellets resulted in similar Ca^{2+} uptake compared to control lysosomes. Since toxins may require intracellular processing, we further wondered whether pre-incubation of lysosomes with cytosol and TT would result in an irreversible augmentation of calcium uptake. The results of these experiments are shown in Table 3. Washed lysosomes that had been pre-incubated with TT and cytosol had statistically higher Ca^{2+} uptake than washed control-lysosomes pre-incubated with cytosol. However, the addition of cytosol and TT (7.5 ug/ml) to the washed lysosomes that had been pre-incubated with TT and cytosol had even higher Ca^{2+} uptake than washed control lysosomes pre-incubated with cytosol. This result is compatible with the following interpretations: (1) inability to completely remove TT when incubated with cytosol; (2) TT with cytosol catalyzes an irreversible reaction that leads to augmented Ca^{2+} uptake; or (3) TT and cytosol together interact with lysosomes in a nonspecific way (e.g. interculation of protein into lysosomal lipid membrane) resulting in augmentation of Ca^{2+} uptake.

To explore the interaction of tetanus toxin with PMN lysosomes, we have directed our efforts to purify the PMN lysosomal Ca^{++}-ATPase. Major progress in the purification of the lysosomal Ca^{++}- uptake pump has been achieved. Utilizing solubilized lysosomal membranes, we have used affinity of the Ca-ATPase for phenothiazines to isolate the pump by phenothiazine-agarose column chromatography (Affi-Gel-phenothiazine, Bio Rad). A partially purified Ca^{++} dependent ATPase was demonstrated in the EDTA-containing eluate (Figure 7, Lanes A, B, C; Lane D: molecular standards; Table 4). From the fraction containing Ca^{++}

dependent ATPase activity, sodium dodecyl sulfate polyacrylamide gel electrophoresis demonstrated five bands with approximate molecular weights of 90,000, 75,000, 58,000, 43,000, and 14,000 (not shown).

Future experiments will be directed to answer the following questions:

1. Does TT modify PKC to a less active form or does TT reduce the quantity of PKC?

2. How does TT mediate the modification or reduction of PKC?

3. Does TT alter the phosphorylation of MO proteins by PKC?

4. Further characterization of the in vivo model.

5. Purification of the Ca^{++} ATPase with the eventual goal of reconstitution of the Ca^{++} dependent ATPase into a lipid vessicle. This would allow us to test if the CA^{++}-dependent ATPase can function as a Ca^{++} uptake pump and to use the isolated enzyme as a target protein for the effect of TT.

LEGENDS TO FIGURES

Figure 1. TT Inhibits Superoxide (O_2^-) generation from human MO.

C-MO and TT-treated MO (1.5 ug/ml) were washed and resuspended in toxin free Hanks Balanced Salt Solution with Ca^{2+} (1 mM) and Mg^{2+} (1 mM). Cells were stimulated with A23187 to produce superoxide at 37°C for 20 min. The cell free supernatant was assayed for superoxide by measuring the amount of cytochrome C reduced. In response to A23187, TT-MO have inhibited O_2^- generation ($p<0.001$, N=14). In comparison to C-MO, TT-treated MO (1.5 ug/ml) generated similar amounts of O_2^- (nM cytochrome C reduced) in response to PMA (30 uM) (C-MO:54.3, TT-MO:51.3), f-met-le-phe (0.5 uM) (C-MO:12.5, TT-MO:14.0) and oponized zymosan (opZymosan; 2 mg/ml) (C-MO:19.9, TT-MO:22.4). The results are the mean, ± SEM of 3-9 experiments.

Statistical analysis was performed using the two-tailed paired Student's t-test, unless otherwise specified.

Figure 2. TT Inhibits O_2^- Generation from Human MO in Response to Multiple Stimuli.

In response to A23187 (1 uM), PMA (30 uM), f-met-leu-phe (0.5 uM) and OpZymosan (2 mg/ml), MO-treated with higher dose of TT (15 ug/ml) produced significantly less O_2^- than C-MO, $^*p < 0.01$, $^+p < 0.01$, $^ap < 0.05$, and $^bp < 0.05$. The results are the mean ± SEM of 4-8 experiments.

Figure 3. Inhibition of Protein Kinase C Activity in TT-Treated Human MO.

3A. PKC activity from cytosolic fraction was significantly diminished in MO treated with TT (1.7 ug per ml), mean ± SEM of 30 separate experiments, $p < 0.0005$. PKC activity of particulate fraction is the mean ± SEM of 10 separate experiments, $p < 0.05$.

3B. Dose-dependent inhibition of PKC activity by TT. The results are the mean ± SEM of 3-7 separate experiments, each compared with at least 2 different doses of TT.

METHODS: Mononuclear cells were isolated from heparinized peripheral blood obtained from healthy volunteers using Hypaque-Ficoll density gradient centrifugation (1,5), washed twice with Hank's balanced salts solution (HBSS) without Ca^{2+} or Mg^{2+}, and resuspended at a concentration of 4-6 x 10^6 cells/ml in RPMI-1640 tissue culture media (GIBCO, Grand Island, N.Y.) supplemented with 10% fetal bovine serum (HyClone Lab., Logan, Utah), 50 U penicillin per ml and 50 ug streptomycin per ml (media). Adherent monocytes were obtained by differential adherence to glass as previously described (1). Adherent monocytes were cultured in supplemented medium with or without TT (lot 505269, CalBioChem, La Jolla, CA). After a 24-40 hour incubation, control (C-) and TT-treated cells were harvested, washed twice with HBSS and resuspended at 2.0 x 10^7 in sonication buffer containing 0.25

M sucrose, 10 mM Hepes (pH 7.5), 2.5 mM EDTA, 10mM mercaptoethanol, 4 mM phenylsulfonylfluoride (PMSF), and 0.05% (w/v) leupeptin (20).
PKC assay: Using the methods of Melloni et al (6) cells were sonicated in a Branson sonicator (model 250). Cell supernatants were obtained by centrifugation at 100,000 g for 30 min at 4°C and the particulate fraction was solubilized with Triton X-100 (0.2%) in sonication buffer. PKC activity was assayed according to a modification (6) of the procedures by Kishimoto et al (7). Phosphorylation of type IIIS bovine histone (0.15-1.5 mg per ml) was performed in buffer containing 50 mM sodium borate (pH 7.5), 5 mM $MgCl_2$, 20 ug PtS per ml, 0.01-1.0 mM $CaCl_2$, 6 uM PMA, ATP (5-10 uM; 1 uM γ-^{32}P-ATP 30 Ci per nmole, New England Nuclear) and 30 ul of cell sonicate in a final volume of 200 ul. In some experiments 10 uM GTP containing γ-P^{32}-GPT 30 Ci per mole, or 10 uM ATP containing α-^{32}P-ATP were used. The reaction was performed in duplicate at 30 C and terminated after 10 min by the addition of 1 ml of 10% trichloroacetic acid. Bovine serum albumin (2 mg) was added as a carrier. Precipitated protein was recovered by filtration (Millipore, HA filter, 0.45 u). Filters were solubilized in Hydroflour (National Diagnostics, Highland Park, N.J.) and counted in a Beckman LS 7000 scintillation counter. Non-specific trapping of P^{32} by TCA precipitable BSA in the absence of cell preparation (enzyme source) was subtracted from all values. PKC activity is defined as phosphorylation in the presence of PtS, Ca^{2+} and PMA (phorbol) minus phosphorylation with these cofactors omitted. Cytosolic phosphorylation with cofactors (PtS, Ca^{2+}, PMA) was 37.7 $\pm$ 6.9 p-mole/10 min for C-MO and 23.2 $\pm$ 4.2 p-mole/10 min for TT-treated (1.7 ug/ml)-MO. Phosphorylation without PtS, Ca^{2+} and PMA from the cytosolic fraction was 6.9 $\pm$ 0.8 p-mole per 10 min for C-MO and 5.7 $\pm$ 0.6 p-mole per 10 min for TT-treated MO, N=30 (Figure 1A). Unless otherwise stated, reagents were purchased from Sigma (St. Louis, MO). Statistical analysis was performed using the two-tailed paired Student's t-test.

Figure 4. Protein kinase A activity from C-MO and TT-treated MO were similar. Concombitant PKC activity from TT-treated MO was 47.8% $\pm$ 10.5% of C-MO. The results are the mean $\pm$ SEM of 4 separate experiments.

Methods. Protein kinase A was assayed according to procedures by Kikkawa et al (11) in buffer containing 20 mM Tris (pH 7.5), 5.0 mM Mg acetate, 1 uM cyclic adenosine monophosphate (cAMP), 150 ug type IIIS histone per ml, ATP/ γ-^{32} P-ATP and 30 ul of cell sonicate in a final volume of 200 ul. Conditions for the reaction and its termination are as described for PKC assay. PKA activity was calculated by subtraction of phosphorylating activity in the presence of 1 mM EGTA and no added cAMP.

Figure 5A. PKC activity in cytosol of C-MO, cells treated with commercial TT (T-CB) or MA State Laboratory tetanus toxin (T-SL). Since commercial TT (T-CB) contained at least four bands (150 to 80 KDa) when examined under non-reducing conditions using 12% sodium dodecylsulfate-polyacrylamide gel electrophoresis (PAGE) and stained by

coomassie blue, we utilized T-SL to examine whether the inhibition of PKC activity was attributable to TT holotoxin. T-SL when examined by the same conditions showed one major band at 150 KDa and a faint band at 140 KDa. Mouse lethality test using methods described previously (1) indicated that T-SL was lethal to C57B6 mice at 0.26 ng per Kg. The results are the mean ± SEM of 3 separate experiments, *, + $p < 0.05$.

Figure 5B. The effect of heat inactivated TT (T-H) and immunoabsorbed TT (T-Abs) on PKC activity expressed as a percent of C-MO PKC activity. The results are the mean ± SEM of 4 separate experiments for T-H and for T-Abs.

Methods. Adherent MO were cultured as previously described in media with or without T-SL (Massachusetts State Laboratory, 4.2 ug per ml) or T-CB (1.74 ug per ml). T-CB (110 ug per ml) was heat inactivated in 2 ml of RPMI 1640 media by heating to 100 C for 1 hour. The macroscopic precipitate was removed by centrifugation at 12,000 g for 1 min. The amount of supernatant from T-H used to treat adherent MO was equivalent to T-CB (1.74 ug per ml). Immunoabsorption of T-CB was performed using anti-TT immunoglobulins bound to protein A-sepharose beads. Protein A-sepharose (400 mg) was swollen and washed with phosphate buffered saline (PBS, pH 7.2). Human anti-TT immunoglobulin (HyperTet, MA State Laboratory, 250 antitoxin units) and 4 ml PBS were added to pre-swollened protein A-sepharose and allowed to bind at room temperature for 1 hour. Hypertet is a polyclonal anti-TT immunoglobulin (Ig) derived from pooled Ig fraction of serum obtained from human donors with high neutralizing titer for TT. The immunoabsorbant was washed 4 times each with 8 ml of PBS and recovered by centrifugation at 200 g for 10 min at 4°C. T-CB (140 ug in 4 ml of RPMI supplemented with 10% fetal calf serum, 50 U penicillin per ml and 50 ug streptomycin per ml) was immunoabsorbed at room temperature for 1 hour. The supernatant was recovered by centrifugation at 200 g for 10 min at 4°C. The amount of supernatant used to treat adherent MO (T-Abs) was equivalent to unabsorbed T-CB at a concentration of 1.74 ug per ml. Cell incubation and PKC assay were as described in the legend for Figure 3.

Figure 6. Female mice (C57B6, 18 grams, Jackson Laboratories) were intraperitoneally injected with 0.1 ml of TT at 10^{-8} mg/ml (Massachusetts State Laboratory) or 0.1 ml of diluent. Serial dilutins of TT were made with RPMI 1640 medium supplemented with 10% fetal bovine serum (diluent). This dose corresponded to a 50 x lethal dose and resulted in generalized tetanus with spastic paralysis at 24 to 36 hours, while lethal dose resulted in death at 60-67 hours. After the development of generalized paralysis, mice were sacrificed by cervical dislocation. Brain and spinal cord were isolated and immediately placed in ice-cold buffer containing 0.25 M sucrose, 10 mM Hepes (pH 7.5), 2.5 mM EDTA and 2.0 mM EGTA. The tissue was transferred to and homogenized in sonication buffer containing 0.25 M sucrose, 10 mM Hepes (pH 7.5), 2.5 mM EDTA, 1 mM EGTA, 2mM phenylsulfonylfluoride (PMSF) and

0.05% (w/v) leupeptin (6). Homogenization was performed on ice using a glass tissue homogenizer. Unbroken cells and tissue were sedimented at 200 g, 4°C for 10 min. The cytosol was separated from plasma membrane vesicles and subcellular organelles (designated as particulate) by centrifugation at 100,000 g for 1 hour. The particulate was solubilized with sonication buffer containing 0.2% Triton-X-100. Assays of PKC from cytosol were performed immediately while the particulate fraction were assayed simultaneously or stored in -70°C for later assay.

The assay for PKC activity were similar to methods reported in Figure 3 with the following modifications. Protein concentration were assayed by BioRad reagent. Cytosol or solubilized particulate preparations from control and TT-treated mice were adjusted to equal protein concentration and 30 ul were used for PKC assay. $CaCl_2$ at 1 mM was added for the PKC assay.

Figure 7. Partial Purification of Ca^{++} ATPase of Lysosomal Membrane

SDS - polyacrylanide gel electrophoresis of fractions which contained high Ca^{2+}-dependent ATPase activity were stained by Coumassie blue. Lanes 1, 2 and 3 from left to right show a band of approximate molecular weight of 90,000. Lane 4 contains molecular weight standards. Using Coumassie blue, one major band was seen, while silver stain of a repeat SDS gel (not shown) demonstrated five bands (approximate molecular weights 90,000; 75,000; 58,000; 43,000; 14,000).

Methods. Lysosomes were isolated from human neutrophils as previously described (17). The lysosomal membranes were isolated by a freeze-thaw method to disrupt the lysosomes. The membranes were centrifuged in an airfuge and the pellet was resuspended in KCl 120 mM, Hepes 30 mM, $MgCl_2$ 2.5 mM, and $CaCl_2$ 100 uM at pH 7.2. Phosphatidylcholine was added to a final concentration of 0.5 mg/ml. The membranes were frozen at -70°C until ready for use. Pooled lysosomal membranes at a known protein concentration (determined by BioRad assay) were solubilized with Triton X-100 (1 mg of Triton X-100 per mg of protein). The nonsolubilized membranes were removed by airfuge centrifugation. The protein concentration of the solubilized membranes was determined by a BioRad assay. Phosphatidylcholine and $CaCl_2$ were added to final concentrations of 0.5 mg/ml and 100 uM, respectively. The solubilized membranes were added to Affi-Gel phenothiazine (a phenothiazine derivative coupled to a crosslinked agarose) which had been mixed with a calcium and phosphatidylcholine-containing buffer. (KCl 130 mM, Hepes 20 mM, $MgCl_2$ 1 mM, $CaCl_2$ 100 uM, dithiothreitol [DTT] 2 mM, Triton X-100 0.4%, phosphatidylcholine 0.05%, pH 7.4). The mixture was placed on a tube rotation for approximately 18 hours at 4°C. The gel mixture was placed on a porous glass filter and washed with 80 mls of the calcium-containing buffer above, then placed in a column and washed with 20 mls (approximately 2 void volumes of an EDTA-containing buffer [KCl 130 mM, Hepes 20 mM, $MgCl_2$ 1 mM, DTT 2 mM, EDTA 2 Mm] Triton X-100

0.4% phosphatidylcholine 0.05%, pH 7.4) 2 ml fractions were collected from the column (0.3 cc/min). Triton X-100 was removed from the individual fractions by BioBeads SM-2 (500 mg BioBeads per 2 ml of samples). $MgCl_2$ and $CaCl_2$ were added to the EDTA-eluted fractions to give final concentrations of 2 mM and 50 uM, respectively. ATPase activity was determined by a coupled reaction assay which measured the disappearance (oxidation) of NADH. 100 ul of samples was added to 900 ul of both a high calcium and low calcium buffer and the change in optical density was measured over 8-10 minutes. The calcium dependent ATPase activity was determined by subtracting the ATPase activity in the low (i.e. 1 mM EDTA) calcium containing buffer from the ATPase activity in the high (i.e. 100 uM) calcium containing buffer. See Table 1 for balance sheet.

Figure 1. TT Inhibits Superoxide Generation from Human Macrophages

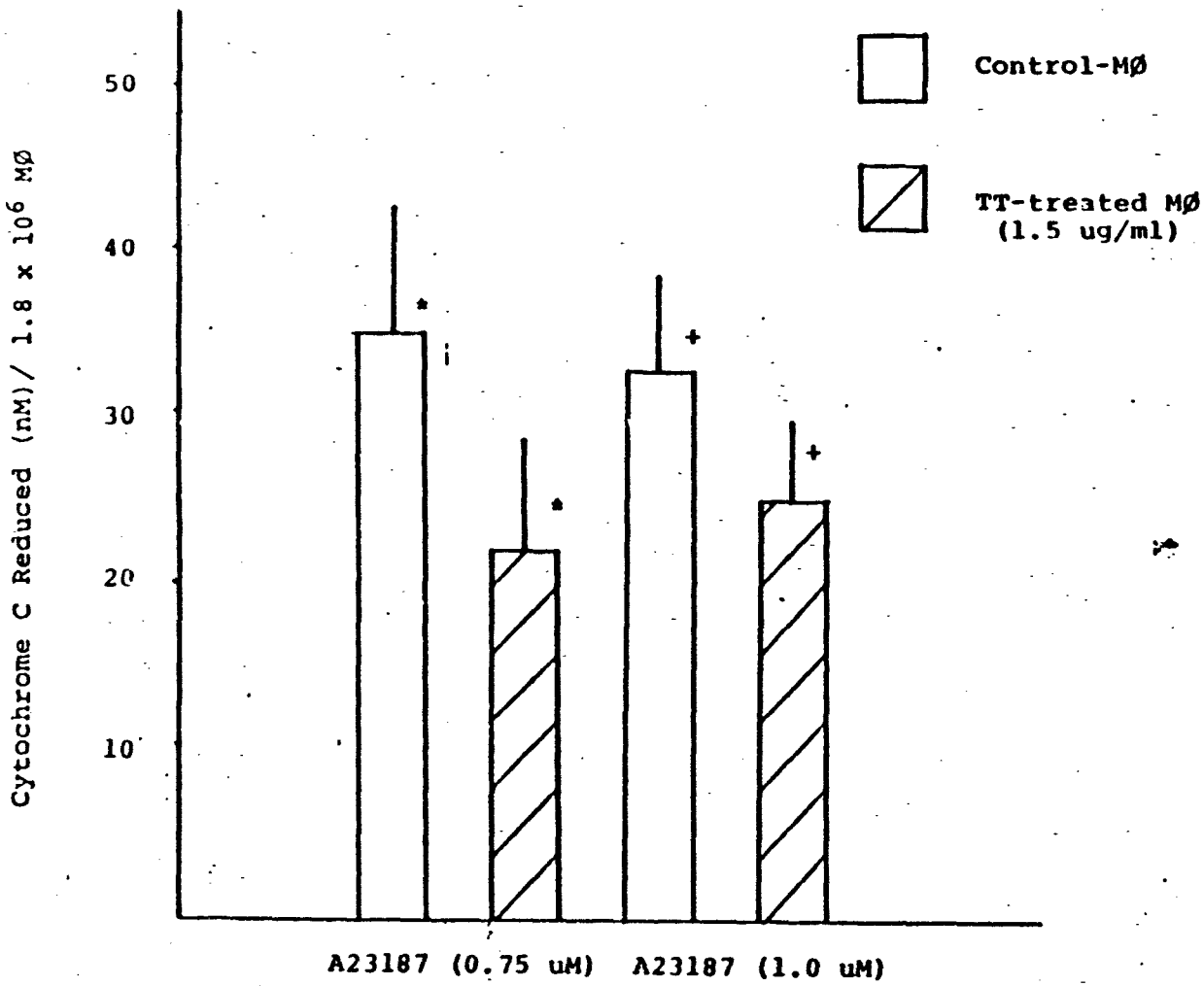

Figure 2. TT Inhibits Superoxide Generation from Human Macrophages

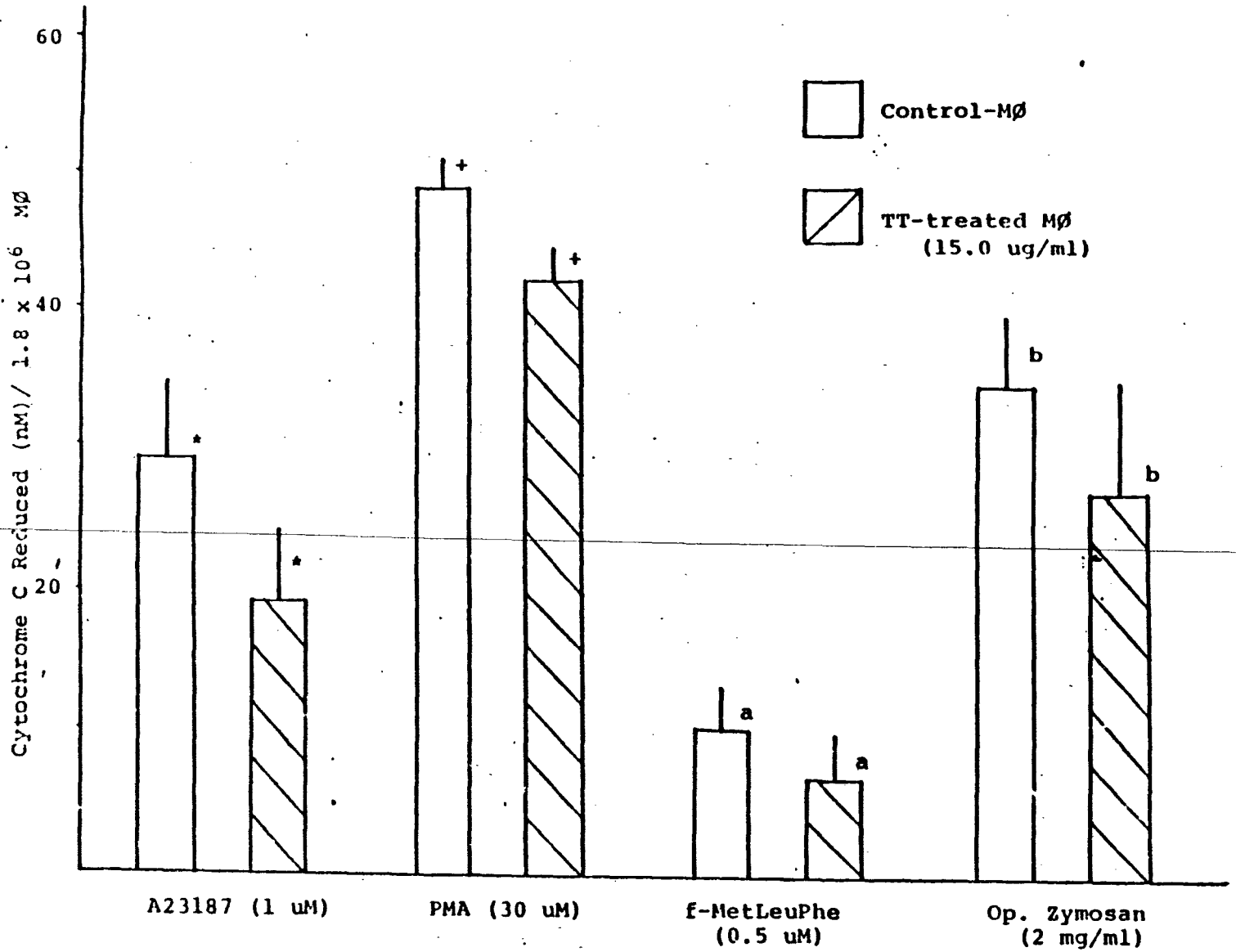

FIGURE 3A

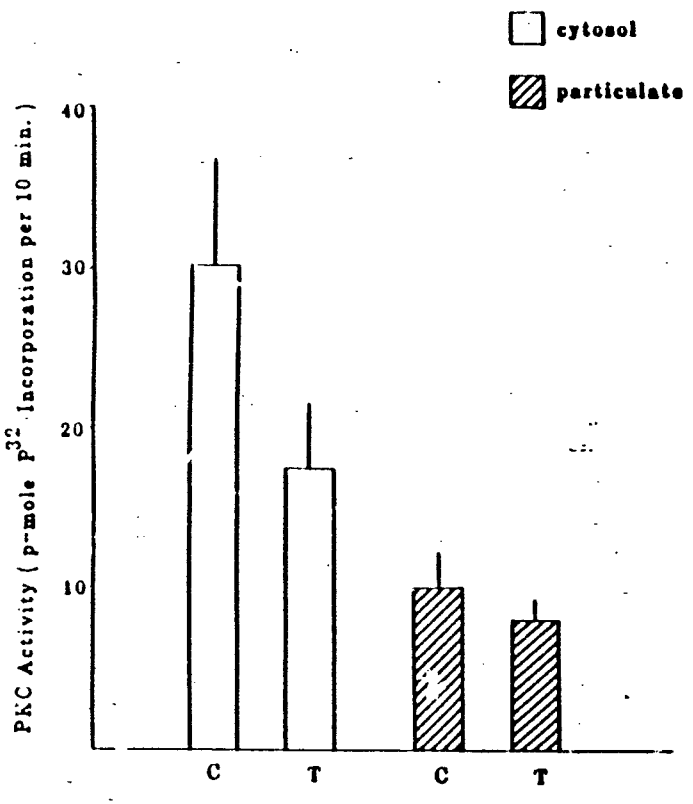

FIGURE 3B

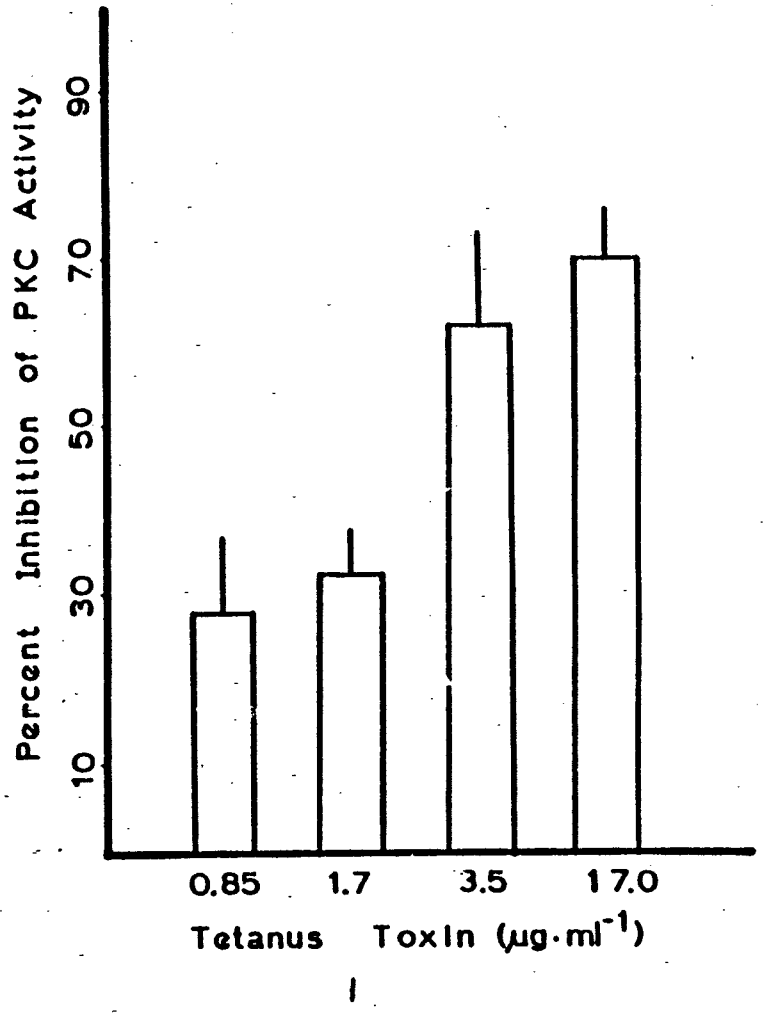

FIGURE 4

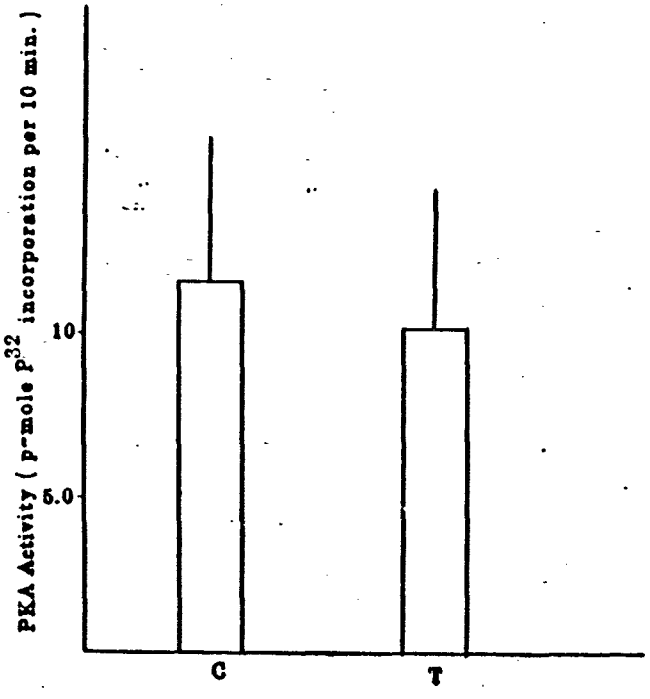

FIGURE 5A

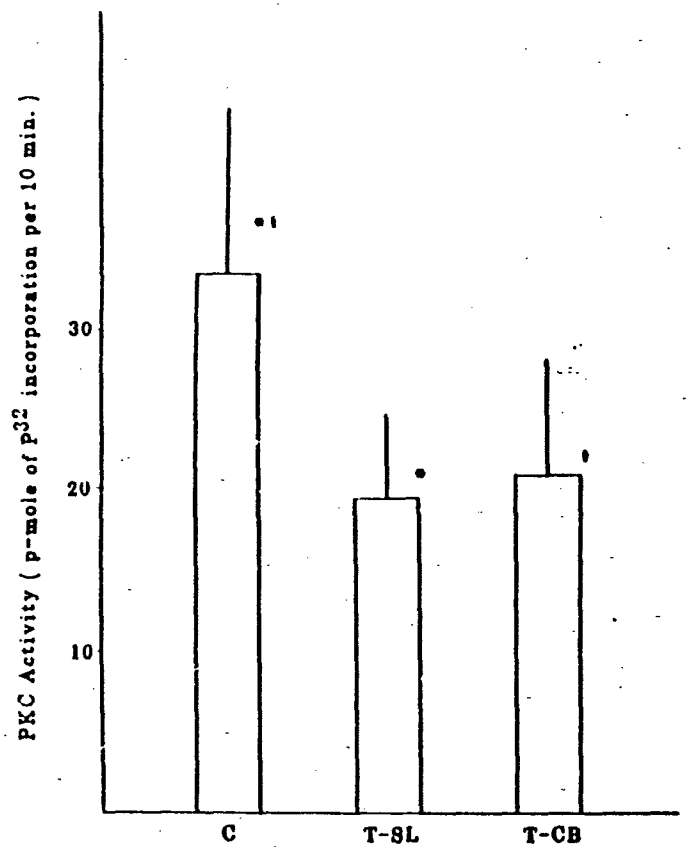

FIGURE 5B

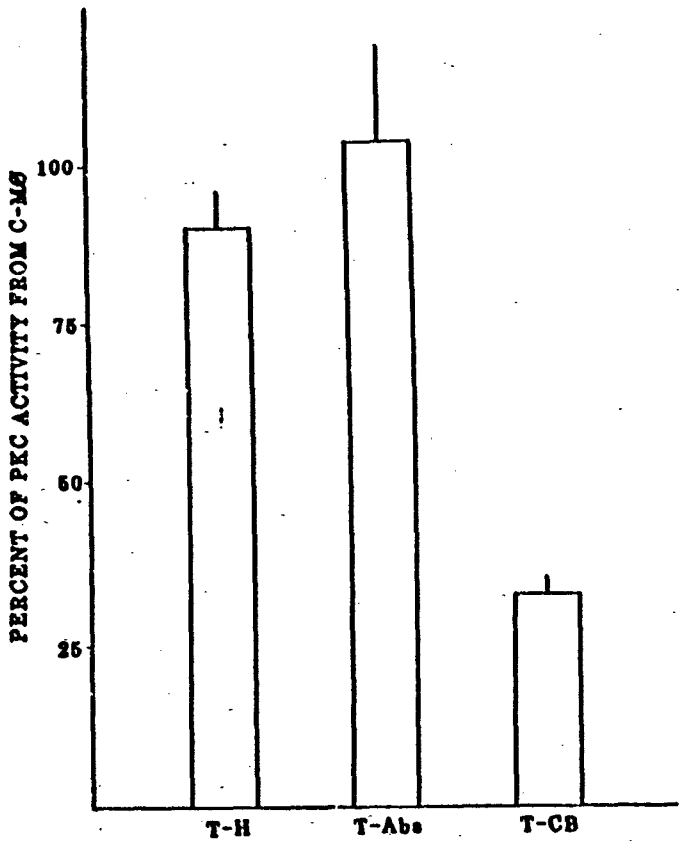

FIGURE 6

DIMINISHED SPINAL PKC ACTIVITY IN CYTOSOL FROM TETANUS INTOXICATED MICE

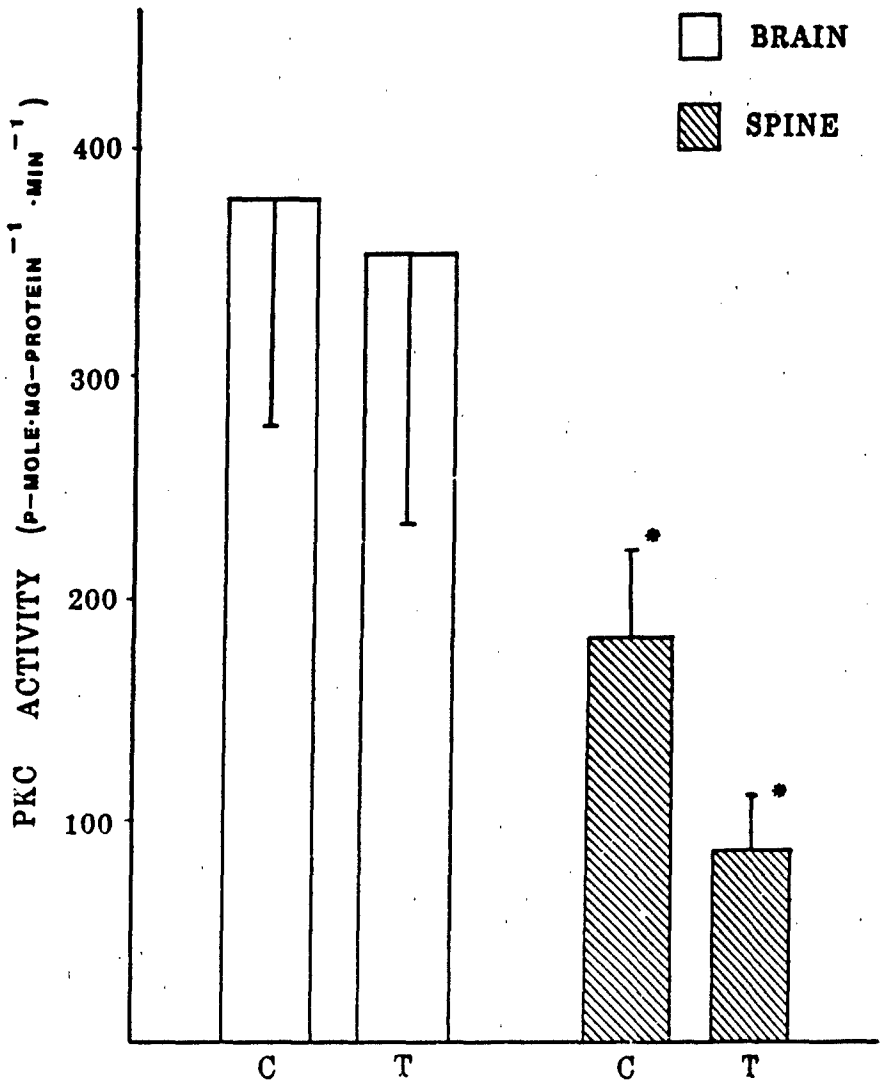

FIGURE 7

LANES: A B C D

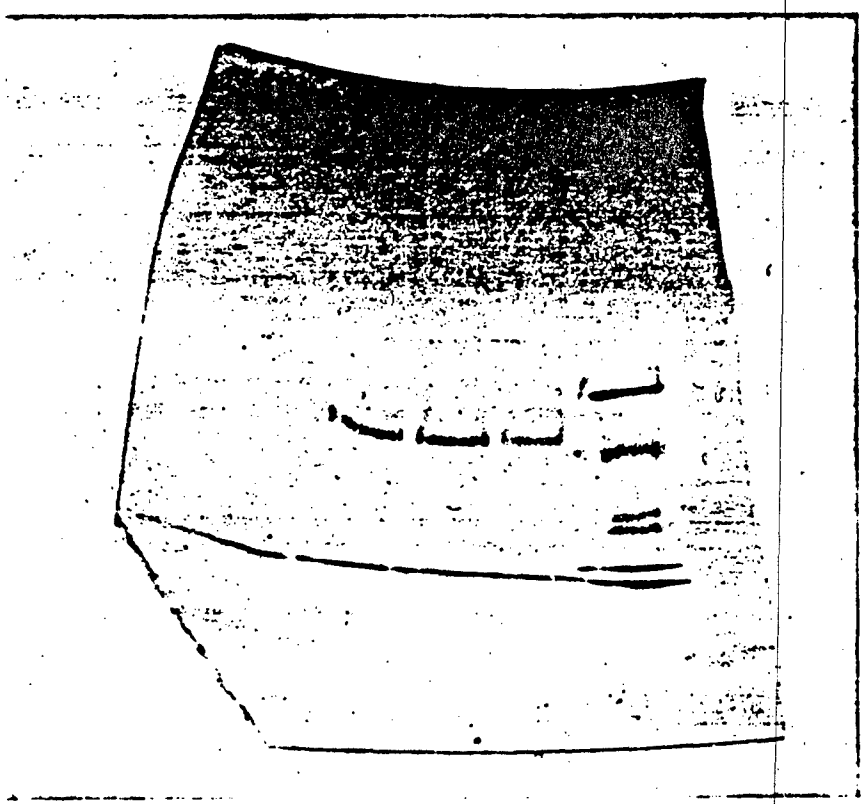

Table 1. The Effect of Different Phorbols on Macrophage Secretion and PKC.

Phorbol	Percent Secretion		PKC Activity	
	Control	TT-treated	Control	TT-treated
α-PPDC 60 uM	3.7 ± 0.4	0.9 ± 0.9	6.2 ± 2.7	4.5 ± 2.6
6 uM	0.3 ± 0.3	0.0 ± 0.0		
β-PDDC 60 uM	28.8 ± 12.8	18.1 ± 8.5	30.9 ± 9.7	13.1 ± 7.9
6 uM	27.0 ± 9.9	11.3 ± 5.6		
PMA 60 uM	44.4 ± 8.5			
6 uM	40.0 ± 12.7	21.6 ± 4.5	39.7 ± 6.2	20.7 ± 5.3

PKC activity is expressed as p-mole P^{32} incorporation per 10 min. The results are the mean ± SEM of 3-5 separate experiments. In a single experiment (data not shown) 60 uM PMA induced only a slightly higher secretion from TT-treated MO. In a single experiment (data not shown) α-PDDC at 6uM did not and β-PDDC at 6 uM did induce a similarly lower PKC activity than PMA at 6 uM.

Methods. Assays of secretion of lysozyme were performed according to previously reported methods (1). The experiments were controlled for the effect of adding dimethlysulfoxide. The percent secretion represents stimulus induced lysozyme release into the supernatant divided by total cellular lysozyme.

Table 2. TT augments Ca^{2+} uptake by human PMN lysosomes

Condition	Time	nmole Ca^{2+}/mg protein
Lysosome	2 min	1.45 ± 0.7+
Lysosome + TT	2 min	2.98 ± 1.2+
Lysosome	5 min	2.41 ± 0.79*
Lysosome + TT	5 min	6.0 ± 1.98*
Lysosome	10 min	4.52 ± 1.16
Lysosome + TT	10 min	7.93 ± 3.24

TT (3 ug/ml, Massachusetts State Laboratory) with lysosomes were suspended in "uptake" buffer containing KCl 100 mM, $MgCl_2$ 5mM, Hepes 20 mM, and $NaCl_2$ 20 mM, and preincubated for 10 min at 37°C. Ca^{2+} uptake was initiated by the addition of prewarmed $CaCl_2$ (100 uM, containing 1 uCi/ml of ^{45}Ca) and prewarmed ATP (1 mM) (17). The results are the mean ± SEM of five separate experiments.

+, * statistical analysis was performed by two-tailed paired Student's t-test, $p<0.05$, N=5.

Table 3. The Effect of TT and Cytosol on PMN-Lysosome Ca^{2+} Uptake

Condition	nmole of Ca^{2+}/mg-protein/5 min
L + cytosol	3.37 ± 0.93[a]
L + cytosol + TT	5.93 ± 0.62[a]
L + cytosol, washed	2.65 ± 0.99[b,c]
L + cytosol + TT, washed	3.64 ± 0.76[b]
L + cytosol + TT, washed, then cytosol + TT readded	5.49 ± 1.36[c]

The results are the mean ± SEM of six separate experiments.
[a,b,c] $p < 0.05$, two-tailed paired Student's t-test.

Methods: PMN lysosomes (L) were prepared by N_2 cavitation (18) and suspended at a final concentration of 200 ug-protein/ml i "uptake" bufer containing KCl 100 mM, $MgCl_2$ 5 mM, Hepes 20 mM, and NaCl 20 mM. Cytosol was prepared by N_2 cavitation of PMN at 4 x 10^7 cell/ml in modified Hanks' media containing $MgCl_2$ 2.5 mM but without added Ca^{2+}. After release from the cavitation bomb, the suspension was collected without EDTA added. The cavitate was subjected to contrifugation (4.5 x 10^5 g/min 4°C). The resultant supernatant was termed "cytosol". Equal volume of cytosol was added to lysosomes to a final lysosomal concentration of 100 ug protein/ml. Lysosome and cytosol with or without TT (7.5 ug/ml, Cal Biochem, Behring Diagnostic) were preincubated at 37°C for 10 min. Lysosomes (in the bottom panel of Table 4) were pelleted by contrifugation (12,000 g, eppendorf). The supernatent was removed; the pellet was washed twice and resuspended in "uptake" buffer. In the last condition on the bottom panel of Table 4, lysosomes were resuspended in "uptake" buffer and cytosol and TT were readded. Ca^{2+} uptake was initiated by the addition of prewarmed $CaCl_2$ (100 uM containing 1 uCi/ml of ^{45}Ca) and prewarmed ATP solution (1 mM). The pH of all solutions were adjusted to pH 7.1. The suspension was agitated and incubated at 37°C. The reaction was terminated after 5 min but spinning through silicone oil. Specific Ca^{2+} uptake were determined as was previously reported (17).

Table 4. Isolation of Ca^{2+}-dependent ATPase from PMN-lysosomes. Ca^{2+}-dependent ATPase activity was demonstrated only in the low calcium eluate from the Affi-Gel phenothiazine column. This fraction contained less than 0.1% of the protein from pooled lysosomal membranes.

BALANCE SHEET

Fraction	Total Protin (ug)	Protein Yield (%)	Ca^{2+}-dependent ATPase Activity (umol/100 ug/min)
Lysosomal membranes	24,300	100	0
Solubilized membranes	5,250	21.6	0
Column Hi Ca^{++} eluate	5,060	20.8	0
Peak EDTA eluate		<0.1	13.88

METHODS: See Figure 7 legend.

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PARTIAL PURIFICATION AND CHARACTERIZATION OF THE HUMAN NEUTROPHIL LYSOSOME CA++-ATPASE. Paul P. Cook*, M. S. Klempner. Dept. of Medicine, New England Medical Center, Boston, MA

We have previously described a novel Ca++-ATPase in neutrophil (PMN) lysosomes. In isolated lysosomes, ATP-dependent Ca++ uptake was inhibited by the phenothiazine trifluoperazine (TFP) (K_i=20 uM). We have now used this characteristic to affinity purify the lysosomal Ca++-ATPase. Human PMN lysosomes were isolated and disrupted by five freeze/thaw cycles. Lysosomal membranes were solubilized with Triton X-100 (1 mg/mg protein) and applied to a phenothiazine affinity column in the presence of Ca++ (0.1 mM). When no further protein eluted from the column, EDTA (2 mM) was added and fractions collected. No Ca++-dependent ATPase activity was detectable in the intact lysosomal membranes, in the solubilized membranes, or in the Ca++ containing eluate. However, after removal of the Triton X-100 with Biobeads, Ca++-ATPase activity (450 umol/100 ug protein/min) was detected only in the low Ca++ eluate. The enzyme activity was completely destroyed by heat (95°C x 1 hour). Activity of this enzyme was not directly affected by calmodulin (5-10 ug/ml) nor by the presence of phosphatidylcholine (0.05%) or phosphatidylserine (0.05%). A single band was present in native-PAGE whereas five bands of approximate molecular weights 90K, 75K, 58K, 43K, and 14K were seen in SDS-PAGE. This is the first description of a Ca++-ATPase isolated from a secretory granule. The isolated PMN lysosomal Ca++-ATPase should be useful for investigating cytosolic substances, other than calmodulin, which regulate this enzyme and which affect the Ca++ dependent functions of PMN.

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LETHAL TETANUS INTOXICATION IS ASSOCIATED WITH DECREASED CYTOSOLIC PROTEIN KINASE C ACTIVITY IN THE SPINAL CORD. J.L. Ho* and M.S. Klempner. Dept. of Medicine, New England Medical Center, Boston, MA.

Tetanus toxin (TT) inhibits secretion from neurons and macrophages (MO). We have previously described that MO cytosolic protein kinase C (PK-C) but not protein kinase A (PK-A) activity is diminished after in vitro treatment with TT. Here we examine an in vivo system to explore whether PKC activity is diminished in mice intoxicated with TT. Mice manifesting generalized tetanus after receiving a 50x lethal dose of TT were sacrificed and PK-C was assayed from homogenized brain and spinal cord by measuring the transfer of γ^{32}P-ATP to histone. PK-C activity (pmole/mg protein/min) was similar in brain cytosol of control (C) and TT-intoxicated mice, (378.2 $\pm$ 107 and 354.4 $\pm$ 119 respectively). Brain membrane associated PK-C activity was also similar for C- and TT-intoxicated mice. Cytosolic PKC activity in the spinal cord was depressed in TT-intoxicated mice, 87.3 $\pm$ 25 compared to control animals, 182.6 $\pm$ 47, $p<.02$, N=7. Membran associated PK-C activity in the spinal cord was similar in C- an TT-intoxicated mice, (21.4 $\pm$ 17.5 vs 20.4 $\pm$ 15.2 pmole/mg protein/min). Cytosolic PK-A activity from brain and spinal cor was similar for C- and TT-intoxicated mice. The ability of TT t interfere with cytosolic PK-C activity in the spinal cord but no the brain may be related to the clinical manifestations of tetanus intoxication.

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Protein Kinase C Activity Is Reduced In Tetanus Toxin Treated Macrophages

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ABSTRACT

Tetanus toxin (TT) is an extremely potent neurotoxin which inhibits neurotransmitter secretion from some neurons (1-10) by an unknown mechanism. Since neurons and phagocytic cells share similar events that couple stimulation to secretion, we have utilized the human macrophage (MO) to study this mechanism. In initial studies we demonstrated that TT inhibits secretion from human MO (11). Because MO secretion in response to phorbol myristate acetate, a stimulus which activates protein kinase C (PK-C) (17), was inhibited by TT (11,18), we wondered whether TT might interfere with PK-C activity. In this report we demonstrate that MO treated with TT have diminished PK-C activity. Protein kinase (PK)-A activity was similar in control (C-) and TT-treated MO. Purified TT as well as commercially available TT diminished PK-C activity while heated TT, which is biologically inert, had no effect on PK-C activity. Moreover, PK-C activity from C-MO and MO treated with a supernatant from which TT had been removed by specific immunoabsorption was similar. These results provide the first evidence that TT interferes with PK-C activity which may be causally related to its inhibition of secretion.

Key words: Tetanus toxin, secretion, protein kinase C, protein kinase A and macrophage.

INTRODUCTION

Tetanus toxin is a potent neurotoxin which is produced by Clostridium tetani. Introduction of as little as 1 ng/kg (1) of the holotoxin, a 150 kDa protein, into laboratory animals causes spastic paralysis and the characteristic syndrome of generalized tetanus (2,3). Investigators have found that TT inhibits neurotransmitter secretion from neurons (1-10) but the molecular basis of TT intoxication remains undefined. Because neurons and phagocytic cells share similar events that couple stimulation to secretion we have utilized the human macrophage (MO) to study this mechanism (11). These similarities include the involvement of phospholipid metabolism, generation of cyclic nucleotides and changes of cytosolic ion homeostasis as part of the events which lead to secretion (12-17). In initial studies we demonstrated that TT inhibited lysozyme secretion from human MO (11). Because MO secretion in response to phorbol myristate acetate (PMA), a stimulus which activates protein kinase C (17), was inhibited by TT and the inhibition of secretion by TT was not directly linked to cytosolic calcium homeostasis (18) we wondered whether TT might interfere with PKC activity. In this report we demonstrate that MO treated with TT have diminished PK-C activity, that this effect is specific, since PK-A was similar in control (C-) and TT-treated MO, and that the diminished PK-C activity is a result of treatment with TT, since treatment with heated-TT or supernatant from which TT was removed by immunoabsorption had no effect on PK-C. These results provide the first evidence that TT interferes with PK-C which may be causally related to its inhibition of secretion.

METHODS

Material Commercial TT (T-CB, lot 505269) was purchased from CalBioChem, La Jolla, CA. Purified TT (T-SL) and human anti-TT immunoglobulin (HyperTet, 250 antitoxin units) were obtained from the Massachusetts State Laboratory. Hanks's balanced salt solution (HBSS) and RPMI-1640 tissue culture media were purchased from Gibco, Grand Island, N.Y. Fetal bovine serum (FBS) was obtained from Hyclone Laboratory, Logan, Utah. Phorbol myristate acetate (PMA, TPA), α-phorbol didecanoate (α-PDDC) and β-phorbol didecanoate (β-PDDC) were purchased from Sigma, St Louis, MO. Stock solutions of phorbol esters (10^{-3} M) were stored in dimethylsulfoxide at -20 C untill use. γ-P^{32}-ATP, α-P^{32}-ATP and γ-P^{32}-GTP were obtained from New England Nuclear. Unless otherwise stated, reagents were purchased from Sigma, St. Louis, MO.

Cell isolation, rupture and fractionation. Mononuclear cells were isolated from heparinized peripheral blood obtained from healthy volunteers using Hypaque-Ficoll density gradient centrifugation (11,19), washed twice with HBSS without Ca^{2+} or Mg^{2+}, and resuspended at a concentration of 4-6 x 10^{6} cells/ml in RPMI-1640 media supplemented with 10 % FBS, 50 U penicillin per ml and 50 ug streptomycin per ml (media). Monocytes were obtained by differential adherence to glass as previously described (11). Adherent monocytes were cultured in supplemented medium with or without TT, heat-inactivated TT or supernatants from which TT has been removed by immunoabsorption. After a 24-40 hour incubation, control (C-) and TT-treated cells were

harvested, washed twice with HBSS and resuspended at 2.0×10^7 in sonication buffer containing 0.25 M sucrose, 10 mM Hepes (pH 7.5), 2.5 mM EDTA, 10 mM mercaptoethanol, 4 mM phenylsulfonylfluoride (PMSF), and 0.05 % (w/v) leupeptin (20). Using the methods of Melloni et al (20) cells were sonicated using a Branson sonifier (model 250). Cell supernatants were obtained by centrifugation at 100,000 g for 30 min at 4 C and the particulate fraction was solubilized with Triton X-100 (0.2%) in sonication buffer.

Protein kinase assay. PK-C activity was assayed according to a modification (20) of the procedures by Kishimoto et al (21). Phosphorylation of type IIIS bovine histone (0.15-1.5 mg per ml) was performed in buffer containing 50 mM sodium borate (pH 7.5), 5 mM $MgCl_2$, 20 ug phosphatidylserine (PtS) per ml, 0.01-1.0 uM $CaCl_2$, 6 uM PMA, ATP (5-10 uM; 1 uM γ - ^{32}P-ATP 30 Ci per mmole, and 30 ul of cell sonicate in a final volume of 200 ul. In some experiments 10 uM GTP containing γ-^{32}P-GTP 30 Ci per mole or 10 uM ATP containing α-^{32}P-ATP were used. The reaction was performed in duplicate at 30 C and terminated after 10 min by the addition of 1 ml of 10 % trichloroacetic acid. Bovine serum albumin (2 mg) was added as a carrier. Precipitated protein was recovered by filtration (Millipore, HA filter, 0.45 u). Filters were solubilized in Hydroflour (National Diagnostics, Highland Park, N.J.) and counted in a Beckman LS 7000 scintillation counter. Non-specific trapping of ^{32}P by TCA precipitable BSA in the absence of cell preparation (enzyme source) was subtracted from all values. PK-C activity is defined as phosphorylation in

the presence of PtS, Ca^{2+} and PMA (phorbol) minus phosphorylation with these cofactors omitted. Protein kinase A was assayed according to procedures by Kikkawa et al (22) in buffer containing 20 mM Tris (pH 7.5), 5.0 mM Mg acetate, 1 uM cyclic adenosine monophosphate (cAMP), 150 ug type IIIS histone per ml, ATP/ - ^{32}P-ATP and 30 ul of cell sonicate in a final volume of 200 ul. Conditions for the reaction and its termination are as described for PK-C assay. PK-A activity was calculated by subtraction of phosphorylating activity in the presence of 1 mM EGTA and no added cAMP.

Secretion Assays of secretion of lysozyme were performed according to previously reported methods (11) using Micrococcus lysodeikticus as the substrate. Cells were suspended at 2×10^7 in HBSS, 1 mM Ca^{2+} and $MgCl_2$ and stimulated for 20 minutes with phorbols at the designated final concentrations. The experiments were controlled for the effect of adding dimethlysulfoxide. The percent secretion represents stimulus induced lysozyme release into the supernatant divided by total cellular lysozyme. The amount of cell death was monitored by the release of cytosolic lactate dehydrogenase and was always less than 8 percent.

TT purity, lethality, inactivation and removal. Commercial TT (T-CB) contained at least four bands (150 to 80 KDa) when examined under non-reducing conditions using 12 % sodium dodecylsulfate-polyacrylamide gel electrophoresis (PAGE) and stained by coomassie blue. T-SL when examined by the same

conditions showed one major band at 150 KDa and a faint band at 140 KDa. Mouse lethality test using methods discribed previosly (11) indicated that T-SL was lethal to C57B6 mice at 0.26 ng per Kg. T-CB (110 ug per ml) was heat inactivated in 2 ml of RPMI 1640 media by heating to 100 C for 1 hour. The macroscopic precipitate was removed by centrifugation at 12,000 g for 1 min. The amount of supernatant from heat-inactivated T-CB (T-H) used to treat adherent MO was equivalent to T-CB (1.74 ug per ml). Immunoabsorption of T-CB was performed using anti-TT immunoglobulins bound to protein A-sepharose beads. Protein A-sepharose (400 mg) was swollen and washed with phosphate buffered saline (PBS, pH 7.2). Human anti-TT immunoglobulin (HyperTet) and 4 ml PBS were added to pre-swollened protein A-sepharose and allowed to bind at room temperature for 1 hour. HyperTet is a polyclonal anti-TT immunoglobulin (Ig) derived from pooled Ig fraction of serum obtained from human donors with a high neutralizing titer for TT. The immunoabsorbant was washed 4 times with 8 ml of PBS and recovered by centrifugation at 200 g for 10 min at 4 C. T-CB (140 ug in 4 ml of RPMI supplemented with 10 % fetal bovine serum, 50 U penicillin per ml and 50 ug streptomycin per ml) was immunoabsorbed at room temperature for 1 hour. The supernatant was recovered by centrifugation at 200 g for 10 min at 4 C. The amount of immunoabsorbed T-CB supernatant (T-Abs) used to treat adherent MO was equivalent to unabsorded T-CB at a concentration of 1.74 ug per ml.

Statistical method. Analysis was performed using the two tailed paired Student's t-test.

RESULTS

PK-C activity, as defined by transfer of P^{32} from γ-P^{32}-ATP to histone in the presence of PMA, added PtS and Ca^{2+} minus phosphorylation without these cofactors, was diminished in TT-treated MO (Figure 1A). The diminished activity could not be explained by redistribution of PK-C to a membrane associated compartment since PK-C activity of the triton X-100 solublized particulate fraction was also diminished in TT-treated MO (Figure 1A). PK-C can be further inhibited by TT in a dose-dependent manner (Figure 1B). For the highest tested dose of TT (17.0 ug/ml), a 70 % inhibition of PKC was observed.

Further evidence that we have detected an inhibition of PK-C activity by TT is provided by the activation of PK-C by different phorbols. PK-C activity in the absence of any phorbol was 6.5 p-mole per 10 min for C-MO and 3.6 p-mole per 10 min for TT-treated MO. α-Phorbol didecanoate (α-PDDC), which does not stimulate the PK-C in other systems (23), does not stimulate macrophage PK-C and does not stimulate lysozyme secretion (Table 1). β-PDDC, which has an intermediate potency to activate PKC in other systems (23), resulted in an intermediate activation of PK-C and lysozyme secretion when compared to PMA at 6 uM (10-fold less). Similar results were observed with TT-treated (1.74 ug/ml) MO (Table 1). In a single experiment (data not shown) 60 uM PMA compared to 6 uM PMA induced only a slightly higher secretion from TT-treated MO but markedly less than C-MO. Also in a single experiment (data not shown) α-PDDC (6 uM) induced insignificant PK-C activity and β-PDDC (6 uM) induced a similarly lower PK-C activity than PMA at 6 uM.

PK-C utilizes the gamma phosphate of ATP to phosphorylate cellular proteins. The TT inhibitable protein kinase activity also demonstrated the same nucleotide specificity. Compared to γ-P^{32}-ATP, phosphorylation utilizing GTP/ γ-P^{32}-GTP was 11.1 % $\pm$ 3.5% and phosphorylation utilizing ATP/ α-P^{32}-ATP was 1.2 % $\pm$ 4.9%, N=4. Similar results were observed for TT-treated kinase activity.

Protein kinase A (c-AMP dependent) activity from cytosolic preparations was similar in C-MO and TT-treated MO (Figure 2). In contrast, cytosolic preparations from the same cells demonstrated diminished PKC activity from TT-treated MO (47.8 % $\pm$ 10.5 % of C-MO).

Since T-CB contained at least four bands when examined by PAGE, we utilized T-SL, a highly purified preparation, to examine whether the inhibition of PKC activity was attributable to TT holotoxin. Experiments intoxicating MO with T-SL also caused diminished PKC activity (Figure 3A). Heat inactivation of TT resulted in loss of biological activity, reversal of its ability to inhibit MO secretion (11) and in similar PKC activity in C-MO and MO treated with heat-inactivated TT (Figure 3B). Moreover, the ability of an active preparation of TT to inhibit MO PKC activity was removed by specific immunoabsorption (Figure 3B).

DISCUSSION

The data presented demonstrate that TT-treated MO have diminished PKC activity and treatment with TT results in a dose-dependent inhibition of PK-C activity. The degree of inhibition of PKC by the highest tested dose of TT is comparable to the ~ 70 % inhibition of PKC observed by Gerard & McPhail et al (22) in neutrophils treated with high concentrations of a kinase inhibitor, C-I. This decreased kinase activity was specific for PK-C since PK-A activity was similar in C-MO and TT-treated MO. Moreover, the diminished PK-C activity is a result of treatment with TT because heat-inactivated TT and removal of TT by immunoabsorption had no effect on PKC. We do not know the mechanism by which TT modifies PKC activity. However, it is not caused by a generalized reduction of protein synthesis since cytosolic protein concentration from C-MO and TT-treated MO did not differ significantly, 273.0 $\pm$ 22.9 ug per 2 x 10^7 cells versus 254.1 $\pm$ 21.1 ug per 2 x 10^7 cells respectively, N=30. When adjusted for protein concentration, PKC (expressed as p-mole/mg-protein/10 min) was 3716.0 for C-MO and 2296.5 for TT-treated (1.7 ug/ml) MO. In addition, increasing the substrate concentration (ATP, histone) had no effect on the diminished PKC activity in TT-treated MO. At low substrate concentration (5 uM ATP, 0.15 mg histone per ml) PKC activity from TT-treated MO was 61.5 % $\pm$ 5.3% of C-MO and with higher substrate concentration (10 uM ATP, 1.5 mg histone per ml) PKC activity from TT-treated MO was 53.1 % $\pm$ 8.9 % of C-MO, N=3.

Protease-activated kinase II (PAK II) is a kinase that

shares with PK-C similar molecular weight and activation by diolein and PtS (25). Although we have not excluded the possibility that our observations are due to inhibition of PAK II, several features make this less likely. Almost all of the reported experiments were performed with 8.5 uM of ATP, an amount which is below the Km of PAK II for ATP (40 uM) (25). Inaddition, compared to no added Ca^{2+}, 1mM Ca^{2} increased kinase activity by 1.3-fold (N=14), a finding which is similar to that reported for PK-C (1.47-fold) but not PAK II (25).

Preliminary experiments suggest that intracellular processing of holotoxin may be required for its ability to inhibit MO PKC activity. Cytosolic preparation from C-MO preincubated with TT holotoxin (0.3 ug per ml) for 5 min prior to initiation of phosphorylation by the addition of ATP/ γ-P^{32}-ATP had similar PKC activity compared to untreated cytosolic preparations (33.9 $\pm$ 0.1 versus 35.6 $\pm$ 0.5 p-mole/ 10 min, N=3). In contrast the cytosolic preparations from TT-treated MO had diminished PKC activity (21.3 $\pm$ 4.5 p-mole/ 10 min). Since PKC appears to be important in stimulus-secretion coupling (17) and has been shown to localize around granules in phagocytic cells from bone marrow (26) and in both neural cell bodies and axon terminals (27), its diminished activity in TT-treated MO may be causally related to the inhibition of secretion by TT.

Microbial toxins have been useful tools in understanding intracellular mechanisms coupling stimulation to cellular response. For example, the use of cholera and pertussis toxins has helped to elucidate the coupling of receptor to adenylate cyclase by regulatory proteins (28, 29). Further characteriza-

tion of the mechanism by which TT inhibits PK-C activity may further our understanding of the role of PK-C in stimulus-response coupling in TT-intoxication and potentially in other systems.

LEGENDS

Figure 1. Inhibition of protein kinase C activity in TT-treated human MO. A. PKC activity from cytosolic fraction was significantly diminished in MO treated with TT (1.7 ug per ml), mean ± SEM of 30 separate experiments, $p < 0.0005$. Cytosolic phosphorylation with cofactors (PtS, Ca^{2+}, PMA) was 37.7 ± 6.9 p-mole/ 10 min for C-MO and 23.2 ± 4.2 p-mole/ 10 min for TT-treated (1.7 ug/ml)-MO. Phosphorylation without PtS, Ca^{2+} and PMA from the cytosolic fraction was 6.9 ± 0.8 p-mole per 10 min for C-MO and 5.7 ± 0.6 p-mole per 10 min for TT-treated MO, N=30 (Figure 1A). PKC activity of particulate fraction is the mean ± SEM of 10 separate experiments, $p < 0.05$. 1B. Dose-dependent inhibition of PKC activity by TT. The results are expressed as a percent of C-MO PKC activity and are the mean ± SEM of 3-7 separate experiments. For these experiments macrophages were treated without or with 2 different doses of TT.

Table 1. Effect of different phorbols on macrophage secretion and PKC activity. The results are the mean ± SEM of 3-5 separate experiments.

Figure 2. Protein kinase A activity from C-MO and TT-treated MO were similar. Concombitant PKC activity from TT-treated MO was 47.8 % ± 10.5 % of C-MO. The results are the mean ± SEM of 4 separate experiments.

Figure 3A. PKC activity in cytosol of C-MO, cells treated with commercial TT (T-CB, 1.74 ug/ml) or MA State laboratory tetanus toxin (T-SL, 4.2 ug/ml). The results are the mean ± SEM of 3

separate experiments, *, + $p < 0.05$. 3B. The effect of heat inactivated TT (T-H) and immunoabsorded TT (T-Abs) on PKC activity expressed as a percent of C-MG PKC activity. The results are the mean $\pm$ SEM of 4 separate experiments for T-H and for T-Abs.

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Figure 1A.

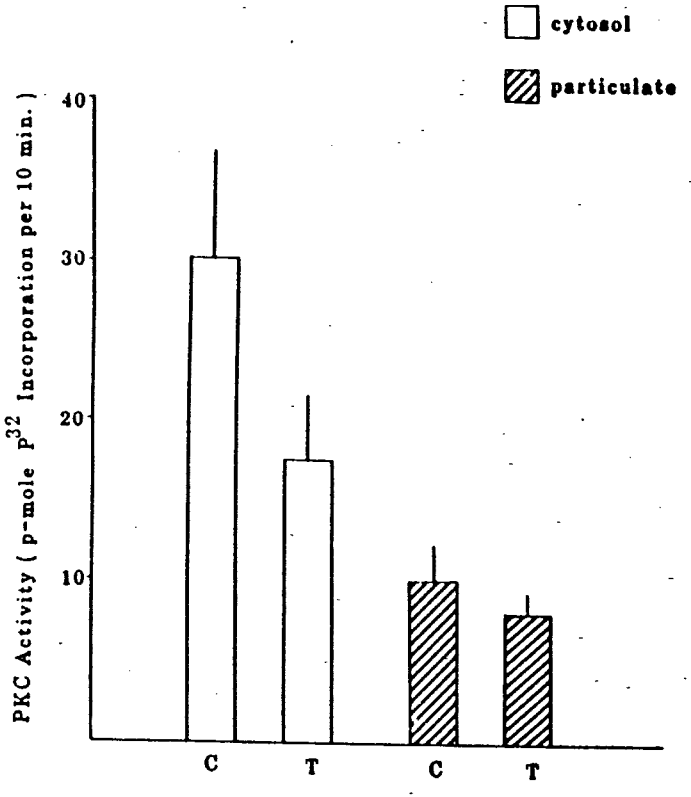

Figure 1B.

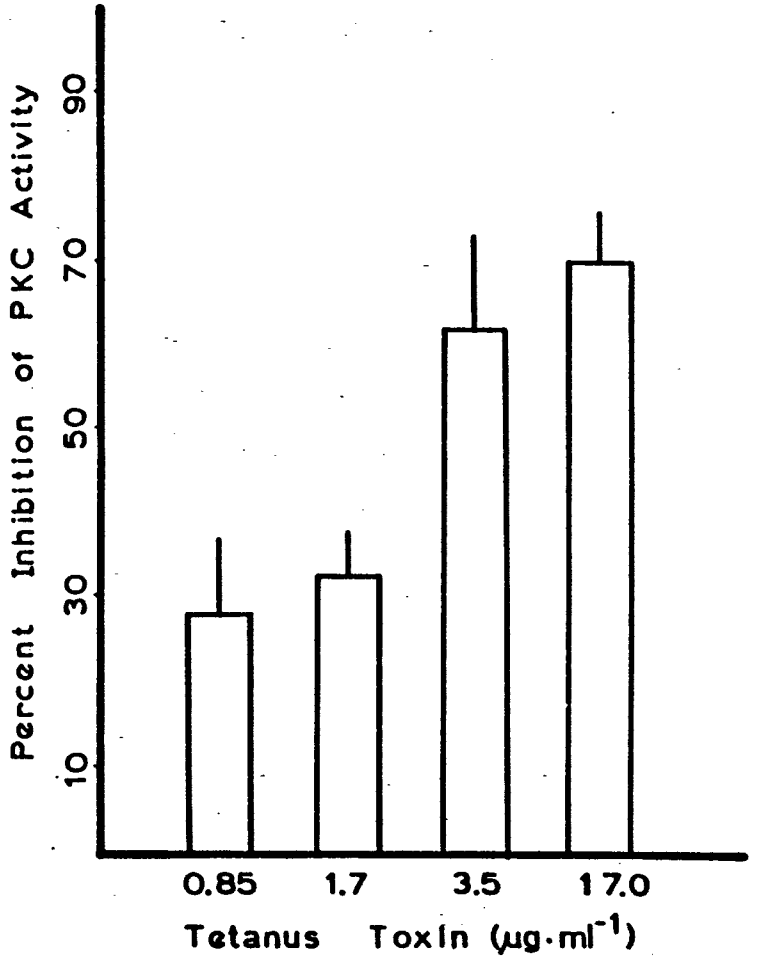

Figure 2.

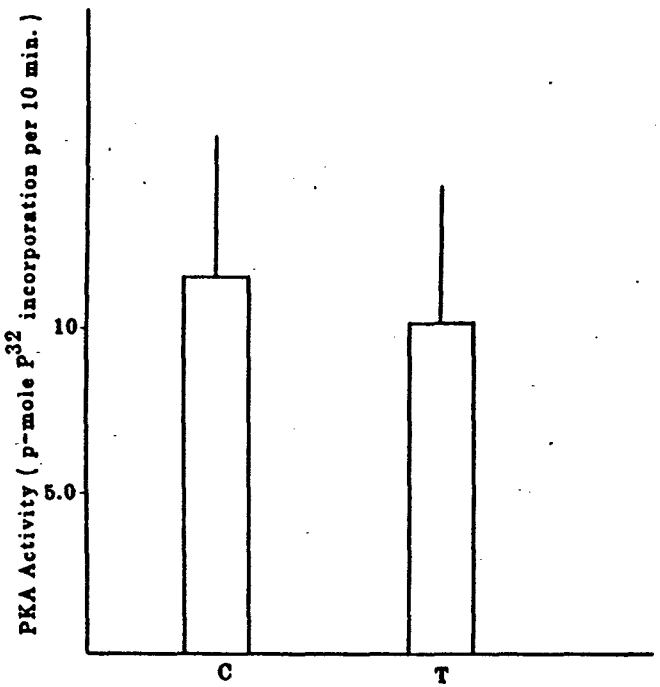

Figure 3A.

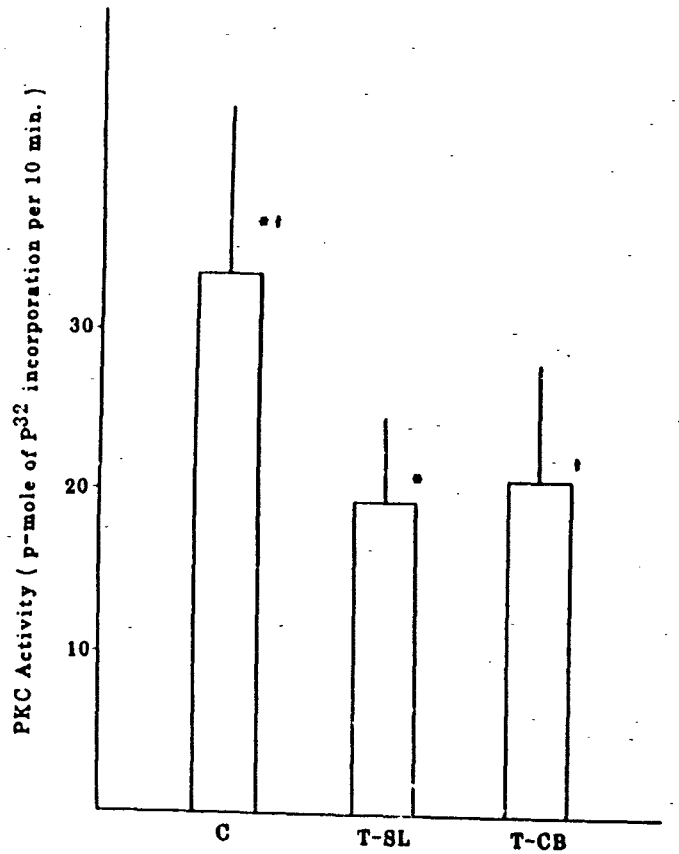

Figure 3B.